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Peptidomic approach identifies Cruzioseptins, a new family of potent antimicrobial peptides in the splendid leaf frog, *Cruziohyla calcarifer*

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KEYWORDS

Cruzioseptins; antimicrobial peptides; peptidomic; molecular cloning; skin secretions; tandem mass spectrometry.

ABSTRACT

Phyllomedusine frogs are an extraordinary source of biologically active peptides. At least 8 families of antimicrobial peptides have been reported in this frog clade, the dermaseptins being the most diverse. By a peptidomic approach, integrating molecular cloning, Edman degradation sequencing and tandem mass spectrometry, a new family of antimicrobial peptides have been identified in *Cruziohyla calcarifer*. These 15 novel antimicrobial peptides of 20–32 residues in length are named cruzioseptins. They are characterized by having a unique shared N-terminal sequence GFLD– and the sequence motifs –VALGAVSK– or –GKAAL(N/G/S) (V/A)V– in the middle of the peptide. Cruzioseptins have a broad spectrum of antimicrobial activity and low haemolytic effect. The most potent cruzioseptin was CZS-1 that had a MIC of 3.77 μ M against the Gram positive bacterium, *Staphylococcus aureus* and the yeast *Candida albicans*. In contrast, CZS-1 was 3 –fold less potent against the Gram negative bacterium, *Escherichia coli* (MIC 15.11 μ M). CZS-1 reached 100% haemolysis at 120.87 μ M. Skin secretions from unexplored species such as *C. calcarifer* continue to demonstrate the enormous molecular diversity hidden in the amphibian skin. Some of these novel peptides may provide lead structures for the development of a new class of antibiotics and antifungals of therapeutic use.

SIGNIFICANCE

Through the combination of molecular cloning, Edman degradation sequencing, tandem mass spectrometry and MALDI-TOF MS we have identified a new family of 15 antimicrobial peptides in the skin secretion of *Cruziohyla calcarifer*. The novel family is named “Cruziioseptins” and contain cationic amphipathic peptides of 20–32 residues. They have a broad range of antimicrobial activity that also includes effective antifungals with low haemolytic activity. Therefore, *C. calcarifer* has proven to be a rich source of novel peptides,

which could become leading structures for the development of novel antibiotics and antifungals of clinical application.

INTRODUCTION

Antimicrobial peptides (AMPs) are a diverse group of oligopeptides that constitute the effector molecules of the innate immune response. They occur in all domains in nature, including bacteria, protozoa, fungi, molluscs, arthropods, vertebrates, and plants. AMPs have a broad spectrum of antimicrobial activity and provide protection against bacteria, fungi, parasites and viruses; however, recent research has provided evidence of additional roles in inflammation, immunity and wound healing [1].

AMPs are extremely diverse in primary structure. There is no clear correlation between structure, potency and selectivity. However, size, charge, hydrophobicity, and amphipathicity are crucial physicochemical properties for their biological activity [1,2]. Most antimicrobial peptides contain between 8–45 amino acids and a positive net charge of +2–+6 at pH7 [3]. In addition, AMPs are usually amphipathic, with a hydrophobic face containing approximately 50% of hydrophobic amino acids. The main mechanism of action involves electrostatic contact of cationic peptides with the anionic membrane of the target microorganisms followed by insertion into the membrane interior. The hydrophobic face interacts with the lipid core while the hydrophilic face interacts with the phospholipids of the cell membrane, and various models have been described, including: carpet-like, toroidal pore, and barrel-stave [1,2]. In addition, some natural AMPs undergo post translational modifications (PTMs) that are required for their antimicrobial function. Common PTMs include: phosphorylation, replacement of L-amino acids with their D-isomers, methylation, amidation, glycosylation, and disulphide bridges [4].

Amphibian skin is one of the richest sources of antimicrobial peptides. Until 2015, around 1600 AMPs had been reported from 165 species and 26 genera [5]. These peptides have been arranged into at least 100 peptide families based on sequence similarities. Remarkably, more than 165 antimicrobial peptides have been reported in the dermaseptin superfamily which occurs in the skins of Central and South American frogs that belong to the Phyllomedusinae subfamily including the genera: *Phyllomedusa* (12 spp.), *Agalychnis* (5 spp.), and *Phasmahyla* (1 sp.) [5–8].

An important characteristic of the members of the dermaseptin superfamily are the highly conserved amino acid sequences in their precursor N-terminal regions that correspond to the signal peptide and acidic spacer peptide. This conservation usually extends to the non-translated regions at the 5' side of the precursor nucleotide sequence. Indeed, the extremely conserved sequences have allowed the design of primers able to target this region and have been instrumental in the discovery of a large number of related peptides. These peptides have been classified in the following families: dermaseptins *sensu stricto*, dermatoxins, phylloxins, phylloseptins, plasticins, medusins, caerin-related peptides and orphan peptides [8–18].

Most studies have been focused on *Phyllomedusa* and *Agalychnis*, while other genera such as *Cruziohyla* remain unexplored. *Cruziohyla* includes two species: *C. calcarifer* that occurs in the Caribbean lowlands from eastern Honduras to the Pacific lowlands of northwestern Ecuador, and *C. craspedopus* that occurs in the Amazon lowlands from Colombia to Peru [19]. *Cruziohyla calcarifer* was recently relocated from the genus *Agalychnis* to the new genus *Cruziohyla* [20] and, considering their taxonomic proximity to *Agalychnis*, it was presumed that this taxon also produce bioactive peptides in their skin.

Several studies have demonstrated the robustness of complementing data from shotgun molecular cloning, Edman N-terminal sequencing and tandem mass sequencing for

peptidomic studies on frog skin secretions [10,21–23]. In the current study, a new family of 15 antimicrobial peptides is reported in the splendid leaf frog, *Cruziohyla calcarifer* and are named cruzioseptins. These contain an N-terminal sequence motif, GFLD– and the sequences –VALGAVSK– or –GKAAL(N/G/S) (V/A)V– in the mid-regions of their mature peptides. Cruzioseptins showed a broad spectrum of antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with low haemolytic effects.

METHODS

Skin secretion extraction

Two adult specimens were collected in northwestern Ecuador (Esmeraldas Province, Durango) in November 2013. Four captive reared sub-adult specimens (from Esmeraldas Province, Reserve Otokiki) were provided in 2015 by Centro Jambatu for Research and Conservation of Amphibians in Ecuador. Skin secretions were obtained after gently massaging the dorsal side of the animals. Secretions were washed off the animals with distilled water. Samples were immediately frozen and stored at -20°C. The frogs collected in the field were returned to their habitat after the extraction. Samples were freeze-dried for analysis in Queen’s University Belfast.

Twelve additional samples were taken from a group of 13-month-old captive bred frogs, whose parental line came from a Costa Rican population. Specimens were housed in terraria as pets in Belgium and Austria. Samples were extracted in the same way as described above, but instead of freeze-dried they were acidified with TFA and were transported at room temperature to the laboratory facilities in Queen’s University Belfast.

Molecular cloning

Lyophilized skin secretions were dissolved in buffer A (99.95% water; 0.05% trifluoroacetic acid), pooled, and aliquoted into two tubes. One was employed for molecular cloning and the other for HPLC fractionation.

One aliquot, equivalent to skin secretion of 2.5 frogs of the Ecuadorian sample, or 1.3 mg of the Costa Rican sample, was dissolved in 1ml of cell lysis/ binding buffer, and polyadenylated mRNA was isolated using magnetic Dynabeads Oligo (dT₁₈) as described by the manufacturer (DynaL Biotec, UK). Isolated mRNA was subjected to 3'-rapid amplification of cDNA by using the SMART-RACE kit (Clontech, UK). In brief, three sets of 3'-RACE reactions were employed. Firstly, 3'RACE used a nested universal primer (NUP) provided with the kit and the sense primer 1 (S1: 5'-CAGCACTTTCTGAATTACAAGACCAA-3') that was complementary to the signal sequence of the phylloseptin-S5 precursor of *Phyllomedusa sauvagii*. Secondly, 3'RACE employed an NUP primer and the sense primer 2 (S2: 5'-TAGACCAAACATGGCTTTCCTGA) designed to target the signal sequence of the first antimicrobial peptide of *Cruziohyla calcarifer* (CZS-1), which was first identified with the primer sense 1 described above. The third 3'RACE included an NUP primer and the sense primer 3 (S3: 5'-AAGAGAGGCTTCCTGGAT-3'), which was also designed based on the sequence of CZS-1 but this time targeting the sequence corresponding to the first 4 amino acids of the mature sequence of the CZS-1 peptide. These primers were designed employing Primer3 and Primer-BLAST online softwares. The 3'-RACE reactions were purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequence.

Reverse-phase HPLC fractionation and Edman degradation

The second aliquot of freeze-dried skin secretion (corresponding to 2.5 frogs) was dissolved in 1.2 ml of buffer A (99.95% H₂O, 0.05% trifluoroacetic acid) and clarified by centrifugation. 1 ml supernatant was subjected to reverse phase HPLC employing Waters Binary pump HPLC system fitted with an analytical column Phenomenex Jupiter C18 (4.6 x 250 mm). Peptides were eluted with a linear gradient formed from 100% buffer A (99.95% H₂O, 0.05% trifluoroacetic acid) to 100% buffer B (80.00% Acetonitrile, 19.95% H₂O, 0.05% trifluoroacetic acid) in 240 min at a flow rate 1 ml/min. Fractions (1 ml) were collected every minute. Detection at 214 and 280 nm was continuous.

Skin secretion of two specimens of *C. calcarifer* from a Costa Rican population was subjected to reverse-phase HPLC using a Diphenyl column C18. Peptides were eluted in a gradient from 1% buffer A (99.95:0.05% H₂O/trifluoroacetic acid) to 80% buffer B (80.00:19.95:0.05% acetonitrile/H₂O/trifluoroacetic acid) in 80 min and fractions were collected every minute. Those fractions were tested for antimicrobial activity and the active fractions 47-53, 59 were re-chromatographed on a Vydac C18 column until clear peaks were obtained. Those samples were sequenced by automated Edman degradation. These analyses were performed in Chris Shaw lab 15 years ago (unpublished data).

MALDI-TOF MS

The molecular masses of peptides and proteins in each chromatographic fraction were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perceptive Biosystems, MA, USA) in positive detection mode employing α -cyano-4-hydroxycinnamic acid matrix. Two microliters of sample plus 1 μ l of matrix (10 mg/ml) were allowed to dry and were later analysed in the range of 500–5000 Da.

1 **Tandem mass spectrometry sequencing**

2 20 µl of the remaining skin secretion fraction were diluted in buffer A and was pumped
 3 directly onto an analytical HPLC column (Phenomenex C-18; 4.6x150 mm) connected to an
 4 LCQ Fleet ESI ion-trap mass spectrometer (Thermo Fisher, San Jose, CA, USA). The linear
 5 elution gradient was formed from 100% buffer A (99.90% H₂O, 0.1% formic acid) to 100%
 6 buffer B (19.9% H₂O, 80% acetonitrile, 0.1% formic acid) in 135 min at a flow rate 20
 7 µl/min. Mass analysis was performed in a positive ion mode with acquired spectra in the
 8 range of m/z 500–2000 with >50% relative intensity during HPLC-MS. Parameters for
 9 electrospray ionization ion-trap mass spectrometry (ESI/MS) were: spray voltage +4.5kV,
 10 drying gas temperature 320°C, drying gas flow 200 µl/min, and maximum accumulation time
 11 –for the ion trap– 350 ms. The first mass analysis was performed in full scan mode, then
 12 peptide ions with >50% relative intensity were selected for fragmentation by collision
 13 induced dissociation (CID), to generate b and y ions that were detected in a second mass
 14 analysis. The instrument was controlled by Xcalibur software (Thermo, USA) and data
 15 analysis was performed using Proteome Discover 1.0 (Thermo, USA). SequestTM algorithm
 16 was employed to compare the acquired fragment ion profiles with the theoretical fragment
 17 ions generated from a FASTA database specific for this species built by molecular cloning
 18 (as described above) to confirm the amino acid sequences of individual peptides.

19 **Solid phase peptide synthesis (SPPS)**

20 Three peptides CZS-1: GFLDIVKGVGKVALGAVSKLF-amide, CZS-2:
 21 GFLDVIKHVVGKAALGVVTHLINQ-amide, and CZS-3:
 22 GFLDVVKHIGKAALGAVTHLINQ-amide were chemically synthesized by solid phase
 23 Fmoc chemistry using a Tribute peptide synthesizer (Protein technologies, Inc). After

cleavage from resin and de-protection, each peptide was purified by HPLC and their degrees of purity were analysed by MALDI-TOF mass spectrometry.

Antimicrobial assays

Antimicrobial screening

500 µl of each HPLC fraction were dried in a vacuum concentrator and later diluted in 10 µl of phosphate buffered saline (PBS). Mueller agar plates with *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* in a 10^6 CFU/ml concentration were prepared and 12 holes were prepared with a sterile Pasteur pipette. 2 µl of each fraction were transferred to one hole of each plate to be tested against the 3 microorganisms. Plates were incubated at 37°C overnight and inhibition zones were recorded as antimicrobial activity.

Minimal inhibitory concentration MIC and minimal bactericidal concentration MBC assays

MICs of the synthetic peptides were determined against *E. coli*, *S. aureus* and *C. albicans*. In brief, serial dilutions of each peptide in dimethylsulphoxide (DMSO) were prepared to obtain concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, 1×10^2 mg/L. Each microorganism in log phase was diluted to obtain the equivalent of 1×10^6 colony forming units (CFU)/ml for the bacteria and 1×10^5 CFU/ml for the yeast. Later, 2 µl of each peptide dilution were transferred to a 96 well sterile plate and 198 µl of the microorganism were added. As controls, 2 µl of DMSO was included instead of peptide and 200 µl of Mueller Hinton Broth in another well. 7 replicates per peptide concentration were performed and the experiment was repeated 3 times in order to confirm the results. Plates were incubated at 37°C for 18–22 h. Growth was monitored at 550 nm in an ELISA plate reader. Later, 10 µl of each concentration without visual growth was sub-cultured on Mueller Hinton agar plates. Plates were incubated at 37°C overnight. MBCs were recorded as the minimal concentration without any growth occurrence.

1 **Haemolysis assay**

2 A suspension of red blood cells (2%) was prepared with defibrinated horse blood (ICS
3 Biosciences) and it was challenged with serial dilutions of the tested peptides resembling the
4 same concentrations employed in the antimicrobial assays previously described. In brief, 200
5 µl of blood cell suspension were incubated with 200 µl of each diluted peptide and they were
6 incubated at 37°C for 120 min. Later, samples were centrifuged and supernatants were
7 transferred to a 96 well plate. Lysis of red blood cells was analysed in an ELISA plate reader
8 at $\lambda=550$ nm. For negative controls, phosphate buffered saline was added to the cells instead
9 of peptide, and for positive controls, phosphate buffered saline with 2% (v/v) Triton X-100
10 (Sigma-Aldrich) was employed. The concentrations that produce 100% haemolysis are
11 reported.

12 **Bioinformatic analysis**

13 Nucleotide sequences were analysed by MEGA6.0 and compared by employing the BLAST
14 tool using databases in the National Centre for Biotechnology Information (NCBI) [24,25].
15 Signal peptides were predicted using the SignalP 4.1 server and theoretical peptide masses
16 were calculated with the peptide mass calculator v3.2 [26,27]. Secondary structure prediction
17 was performed using the GOR4 programme and the physicochemical properties of the
18 peptides were calculated using HeliQuest Computational Parameters and Peptide property
19 calculator Bachem [28-30].

20 **RESULTS**

21 **Molecular cloning of novel antimicrobial peptide precursor-encoding cDNAs**

22 Seven full-length and four partial-length cDNAs encoding novel peptides were cloned from
23 the cDNA library that was constructed from the skin secretion of *Cruziohyla calcarifer*

(Table 1 and Figure 1). The novel peptides are named Cruzioseptins (CZS) to represent their origin in *Cruziophyla* –a genus in honour of a Brazilian herpetologist, Carlos Alberto Gonçalves da Cruz, in recognition of his various contributions to knowledge of Phyllomedusinae [20]. The open reading frames of these sequences contained 195–231 nucleotides. Translated amino acid sequences revealed that the precursors consisted of: (1) a putative signal peptide of 22 residues; (2) an acidic spacer peptide of 23 residues containing 2 pro-peptide convertase processing sites; and (3) a mature peptide of 20–32 residues (Figure 1). In addition, 6 of the 15 peptides were C-terminally amidated with a Gly (G) residue as the amide donor (Table 2). Nucleotide sequences were submitted to the GenBank (NCBI) under accession numbers: KX065078–KX065088.

Each novel nucleotide sequence was analysed using the NCBI database and they showed 80–91% similarity with dermaseptins from *Phyllomedusa hypochondrialis* (Accession number AM229015.1), *Agalychnis annae* (Accession number AJ005187.1), and *P. bicolor* (Accession number Y16564.1). In addition, the BLAST/p (protein/protein) comparisons using only the translated mature sequences of these peptides, showed a lower similarity (45–90%) with dermaseptins. For example: CZS-4 was 45% similar to dermaseptin-B6 from *P. bicolor* (accession number AFR78287.1), CZS-6 was 65% similar to dermaseptin SVII from *P. sauvagii* (accession number CAD92230.1), and CZS-8 was 90% similar to dermadistinctin-L from *P. distincta* (accession number P83639.1). However, when the translated amino acid sequences of the mature peptides CZS-1 and 15 were subjected to BLAST/p analysis, no significant hits were found, not with any amphibian skin antimicrobial peptide or with antimicrobial peptides from other sources.

Edman degradation sequencing

Cruzioseptins 10–15 were found first by antimicrobial activity screening of reverse phase HPLC fractions of *C. calcarifer* skin secretions from the Costa Rican population. Peptides were re-chromatographed for purification and sequenced by Edman degradation. The sequences are shown in Table 2. Later, two of them were cloned from the same population, but none were cloned from the Ecuadorian population to date. The peptide sequences were submitted to the UniProt Knowledgebase under accession numbers: C0HK07- C0HK012.

Isolation and structural analysis of cruzioseptin

During functional screening of HPLC fractions of the skin secretion of *C. calcarifer*, antimicrobial activity against *S. aureus* and *C. albicans* was identified in fractions 162, 163, 171 and 172 (Figure 2). Cruzioseptin-1 was identified in HPLC fractions 171 and 172 based on its monoisotopic molecular mass $[M+H]^+ m/z$ of 2117.54 as determined by MALDI-TOF mass spectrometric analysis and confirmed by LCQ ESI MS full scan that revealed ions $2+ m/z$ 1059.75 and $3+ m/z = 706.67$ (Figure 3). In addition, cruzioseptin-2 was identified in HPLC fractions 162 and 163 due to its monoisotopic molecular mass $[M+H]^+ m/z$ of 2427.42, as found by MALDI-TOF and confirmed by a LCQ ESI MS full scan, where ions $2+ m/z = 1215.08$, $3+ m/z = 810.50$, and $4+ m/z = 316.25$, were identified (Figure 4).

It is remarkable that all cruzioseptins 1 to 15 were 100% identified by LCQ MS/MS fragmentation sequencing employing the whole skin secretion of *C. calcarifer* (Table 2).

Antimicrobial and haemolytic assays of cruzioseptins

Once sequences were confirmed, cruzioseptins 1-3 were selected for further analysis. CZS 1 and 2 were chosen because these peptides were identified in HPLC fractions as detailed above, but in order to determine their potency and specificity more pure peptides were

required. CZS-3 was included later due to the sequence similarity with CZS-2, aiming to determine the effect of the 3 amino acid differences in its antimicrobial activity.

Cruzioseptins 1, 2 and 3 were synthesized by solid phase Fmoc chemistry, purified by HPLC, and the sequences were confirmed by LCQ MS/MS sequencing (Figure 5 and 6). Physico-chemical properties of CZS1–3 are summarized in Table 3. Synthetic pure peptides were employed in antimicrobial and haemolytic assays. Cruzioseptin-1 displayed potent broad-spectrum antimicrobial activity against all three microorganisms tested with MICs of 15.11 μ M against *E. coli* and 3.77 μ M against *S. aureus* and *C. albicans*. In addition, the MBC was below 15.11 μ M for the three microorganisms. At the antimicrobial concentration of 3.77 μ M, this peptide showed only 1% haemolytic activity while reaching 20% haemolysis at 15.11 μ M. CZS-1 reached 100% haemolysis at 120.87 μ M. In addition, cruzioseptin-2 showed moderate broad-spectrum antimicrobial activity against *E. coli* (MIC of 26.35 μ M), *S. aureus* (6.59 μ M), and *C. albicans* (13.18 μ M). The MBC concentrations were below 52.69 μ M. for the three microorganism. Nevertheless, haemolytic activity at 13.18 μ M was only 26% reaching 100% haemolysis at 210.96 μ M. In contrast, synthetic cruzioseptin-3 was less potent than CZS-1 and CZS-2 showing MICs of 13.32 μ M against the three microorganisms tested. Moreover, the MBC was similar to CZS-2 (53.31 μ M). However, haemolysis at this concentration was only 6%. CZS-3 produced 100% haemolysis at 213.33 μ M. Results of these tests are summarized in Table 4 and Figure 8.

DISCUSSION

Antimicrobial peptides secreted by phyllomedusine frog skins are extremely diverse. At least eight families of antimicrobial peptides have been reported so far. These peptides have been classified based on similarities of their primary structures and/or structural motifs. The most

diverse family is the dermaseptins *sensu stricto*, which contains more than 75 peptides described from 15 species [8].

Through a combination of molecular cloning, Edman degradation sequencing, and LCQ tandem MS/MS, a new family named ‘cruzioseptins’ of 15 antimicrobial peptides were found in the splendid leaf frog, *Cruziohyla calcarifer*. All these novel peptides share these unique structural sequences: (1) the N-terminal motif GFLD–; and (2) the motif –GKAAL(N/G/S)(V/A)V– or –VALGAVSK–. In fact, 13 of the cruzioseptins (CZS-2 to CZS-14) present the motif –GKAAL(G/N/S)(V/A)V– and 2 cruzioseptins (CZS-1 and 15) present the motif –VALGAVSK– (Table 2). Their precursor sequences are extremely conserved, sharing high similarity in the signal and acidic spacer sequences at the N-terminal ends but showing important variation in the mature sequences at their C-terminal ends. A BLAST/n search in the NCBI database identified the precursor sequences of these peptides as members of the dermaseptin superfamily. In addition, the BLAST/p comparisons with the translated mature sequences of these peptides, showed 45–90% similarity to dermaseptins. However, CZS-1 and CZS-15 did not produce any significant hits when compared with BLAST/p, suggesting that these were a well differentiated group of peptides that we recognize as a new family based on having a set of unique shared structural motifs and sequences. With a closer analysis of CZS-8, 11 and 14 sequences, it was found that the similarities with dermaseptins were concentrated in the centre of the mature peptides where these cruzioseptins share the dermaseptin motif –AAGKAALNV–. However, all cruzioseptins lack the characteristic Trp (W) in position 3 of dermaseptins. For that reason, and for having the motif GFLD– at their N-terminals, and the motifs –GKAAL(N/G/S)(V/A)V– or –VALGAVSK– at the mid-region, these novel antimicrobials were not classified as dermaseptins; instead, they were assigned to a new family of antimicrobial peptides – the cruzioseptins.

The GFLD– N-terminal motif is also found in other four amphibian skin antimicrobial peptides, including: ranatuerin-3 from *Rana catesbeiana* (accession number P82780.1), brevinins 2PTd and 2Pte from *Pulchrana picturata* (accession numbers POC8T6.1 and POC8T7.1, respectively), and frenatin-4 from *Litoria infrafronata* (accession number P82023.1). These species belong to the families Ranidae and Hylidae. However, neither ranatuerin, brevinin or frenatin families contain GFLD– as a specific motif, so their appearance in these families is most likely a result of convergent evolution. On the other hand, the strongly-conserved nucleotide precursor sequences of cruzioseptins at their N-terminals in common with other members of the dermaseptin superfamily, such as litorins and caerin of the Australian frogs of the Pelodryinae subfamily, supports the view that the genetic origin of the ancestral gene precursor of cruzioseptins was present in the common ancestor which originated prior to the fragmentation of Gondwana. In addition, the extraordinary diversity of cruzioseptins found in a single species provides evidence, once again, that evolutionary mechanisms such as hypermutability of the C-terminal domain, gene duplication, and diversifying selection can provide a wide range of antimicrobial protection [9,31].

In addition, three cruzioseptins were chemically synthesized and their antimicrobial profiles were analysed, showing that all three cruzioseptins (CZS1–3) have broad spectra of antimicrobial activity and relatively low haemolytic activity (Table 4). Firstly, CZS-3 showed potent activity (MIC) against the Gram negative bacterium *E. coli* at 13.32 μ M, followed by CZS-1 at 15.11 μ M and CZS-2 at 23.35 μ M. In addition, at these concentrations, the peptides presented relatively little haemolysis (6%, 9%, and 26%, respectively) (Table 4 and Figure 8). However, in comparison with other antimicrobial peptides of similar sequences (50–70% similarity) such as dermaseptin-B4 from *P. bicolor* (accession number P81486) and dermadistinctin-L from *P. distincta* (accession number P83639), cruzioseptins are less potent

than dermaseptins, whose MICs are 5 and 2.5 μM , respectively (Table 4). Secondly, CZS-1 was the most potent of the three cruzioseptins, being able to inhibit the growth (MIC) of the Gram positive bacterium *S. aureus* at 3.77 μM ; to achieve the same goal, CZS-2 is 2-fold less potent and CZS-3 is 3-fold less potent. However, dermaseptin-B4 and dermadistinctin-L are still more potent (MICs 3.0 μM and 1.3 μM , respectively) [32,33]. Finally, CZS-1 was also able to inhibit the growth (MIC) of the yeast *C. albicans* at 3.77 μM while CZS-2 and CZS-3 needed 3-fold this concentration to achieve the same goal (Table 4). Cruzioseptins 1-2 were bactericidal against *E. coli* having the same MIC and MBC concentrations. However, cruzioseptins 1-3 have a bacteriostatic effect against *S. aureus* and *C. albicans*, requiring a two or three folds concentration increase to reach the bactericidal effect (Table 4). This is an important result because there are relatively few peptides that exhibit antifungal activity and the need to develop new antifungal agents is always growing. The differences in activity found between CZS-2 and CZS-3 are very interesting because these peptides are very similar in their primary structures (87%) and both have a charge of +2. They differ only in 3 amino acids: I/V in position 6, V/I in position 9 and V/A in position 16 (Table 3).

The predicted secondary structures and physico-chemical properties of the three cruzioseptins (CZS-1, CZS-2, and CZS-3) are shown in Table 3. All three cruzioseptins have a similar hydrophobicity (H value range 0.523–0.581) and hydrophobic moment (0.441–0.472 μH), although the primary structure of CZS-1 compared to CZS-2 and CZS-3 is different sharing only 12 conserved amino acids (57%). In addition, CZS-1 has a predicted helical domain containing 19.05 % of the peptide that increases to 30.43% for CZS-3 and decreases to 0% for CZS-2. Moreover, CZS-1 possesses a higher net positive charge than CZS-2 and CZS-3 (+3 versus +2). Helical wheel plots showed that all three cruzioseptins are amphipathic having 11–13 amino acids placed in the hydrophobic face (V/ I/ A/ F/ L/ G) and 8–12 amino acids hydrophilic residues placed at the opposite side (Table 3 and Figure 7).

These 3 variations in sequence change the potency of CZS-2 making it weaker than CZS-3 against *E. coli* (13.33 vs 26.35 μM) but more potent against *C. albicans* (13.33 vs 6.59 μM).

In summary, the antimicrobial potency observed for CZS-1 could be due to its +3 charge, in contrast to the +2 of CZS-2 and CZS-3. However, in comparison with other antimicrobial peptides such as dermaseptin-B4 and dermadistinctin-L, CZS-2 is weaker against *E. coli*, but potent against *S. aureus*. Moreover, CZS showed potency against *S. aureus* and *C. albicans* with only 1% haemolysis at those concentrations, which makes CZS-1 an interesting peptide and warrants further study into its potential antibiotic and antifungal functions.

In conclusion, cruzioseptins, a novel antimicrobial peptide family, is reported in *Cruziophylla calcarifer*. Three synthetic cruzioseptins displayed broad-spectrum antimicrobial activity against *S. aureus*, *C. albicans* and less potently against *E. coli* with minor haemolytic activity. These data show once again, the phenomenal peptide diversity produced in the skin of phyllomedusine frogs such as the previously unstudied *C. calcarifer*. Interplay between molecular cloning and tandem mass spectrometry sequencing, together with functional studies of natural and synthetic peptides have proven to be a robust, cost-effective strategy for peptidomic analysis in species where databases are not available. In addition, these techniques are sensitive enough to generate data with only a few milligrams of material, and this is especially beneficial in the analysis of endangered species where samples are limited. Finally, the discovery of novel natural antimicrobial peptides such as cruzioseptins is a key element in the development of new therapeutic drugs based on the structures of natural compounds.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORSHIP

- 1 This study was conceived and designed by CS, MZ, TC. Sample collections were performed
- 2 by CPB and LAC. Data were acquired by CPB. LC-MS/MS analysis was performed by LW.
- 3 The article was written by CPB and reviewed critically by CS and LAC.

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Table 1. Antimicrobial peptides of *Cruziophyla calcarifer* identified by molecular cloning.

Peptide	Signal peptide	Acidic spacer
	1 * * * * *	* * * * *
CZS-1 (14)	MAFLKKSLFLVLFLGLVSLSI	CEEEKREEE-NEEEEQDDDEQSEEKR
CZS-2 (2)	MAFLKKSLFLVLFLGLVSLSI	CEEEKREEEENEEVQEDDDQSEEKR
CZS-3 (1)	- - - - -	- - - - - KR
CZS-4 (1)	- - - - -	- - - - - KR
CZS-5 (1)	- - - - -	- - - - - KR
CZS-6 (2)	MAYLKKSLFLVLFLGLVSLSI	CEEEKREEEENEEQEDDDQSEEKR
CZS-7 (4)	MAKLKKSLFLVLFLGLVSLSI	CEEEKREEEENEEVQEDDDQSEEKR
CZS-8 (6)	MAFLKKCLFLVLFLGLVSLSI	CEEEKREEEENEEVQEDDDQSEEKR
CZS-9 (1)	- - - - -	- - - - - KR
CZS-11 (6)	MVKLKKSLFLVLFLGLVSLSI	CEEEKREEEENEEVQEDDDQSEEKR
CZS-12(10)	MAFLKKSLFLVLFLGLVSLSI	CEEEKREEEENEEVQEDDDQSEEKR
	Mature peptide	
	46 * * * * *	* * * * *
CZS-1 (14)	GFLDVI - - - KGVGKVALGAVSKLFGQEEER*	-
CZS-2 (2)	GFLDVI - - - KHVGKAAALGVVTHLINQGEQ*	-
CZS-3 (1)	GFLDVV - - - KHIGKAAALGAVTHLINQGEQ*	-
CZS-4 (1)	GFLDVI - - - KHVGKAAALSVVSHLINEGEH*	-
CZS-5 (1)	GFLDVIKHVGKAVGKAAALNAVNDMVNKPQQQS	-
CZS-6 (2)	GFLDVI THVGKAVGKAAALNAVTEMVNAEQ*	-
CZS-7 (4)	GFLDVVKHVGKAVGKAAALNAVTEMVNAEQ*	-
CZS-8 (6)	GFLDVIKHVGKAAAGKAAALNAVTEMVNAEQ*	-
CZS-9 (1)	GFLDVI THVGKAVGKAAALNAV NEMVNAEQ*	-
CZS-11 (6)	GFLDVIKHVGKAAAGKAAALNAVTEMVNAEQ*	-
CZS-12(10)	GFLDVVKHVGKAVGKAAALNAVNDLVNAEQ*	-

*Conserved sites, (x) number of clones with the same sequence. Accession numbers: KX065078-KX065088, COHK07-COHK-12.

Table 2. Amino acid sequences of cruzioseptins confirmed by tandem mass spectrometry sequencing. Characteristic motifs of cruzioseptins are highlighted. Amidation was predicted according to the precursor sequence.

Peptide	Origin	Sequence	Identify by	Coverage %	# Peptides fragments	#AAs	LCQ MW [Da]	Theoretical average mass Da.	Score
CZS-1	ECU	GFLDVIKGVGK VALGAVSK LFa	- - - - - mc, ms ²	100	110	21	2117.26	2117.60	122.74
CZS-2	ECU	GFLDVIKHV GKAAALGVVTHLINQ a	- - - - - mc, ms ²	100	92	23	2428.40	2428.90	43.61
CZS-3	ECU	GFLDVVKH IGKAAALGAVTHLINQ a	- - - - - mc, ms ²	100	64	23	2400.36	2400.85	10.92
CZS-4	ECU	GFLDVIKHV GKAAALSVVSHLINE a	- - - - - mc, ms ²	100	66	23	2445.37	2445.89	23.22
CZS-5	ECU	GFLDVIKHVGKAV GKAAALNAV NDMVNKPQQQS	mc, ms ²	100	155	32	3376.79	3378.90	107.32
CZS-6	ECU	GFLDVI THVGKAV GKAAALNAV TEMVNAEQ*	- mc, ms ²	100	154	30	3109.62	3111.57	90.29
CZS-7	ECU	GFLDVVKHVGKAV GKAAALNAV TEMVNAEQ*	- mc, ms ²	100	119	30	3122.65	3124.61	70.27
CZS-8	ECU	GFLDVIKHVGKAA GKAAALNAV TEMVNAEQ*	- mc, ms ²	100	117	27	2780.50	2781.28	46.11
CZS-9	ECU	GFLDVI THVGKAV GKAAALNAV NEMVNAEQ*	- mc, ms ²	100	101	27	2794.48	2795.26	38.79
CZS-10	CR	GFLDVLKGV GKAAALGAVTHHIN NLVNQQ*	- Ed,ms ²	100	120	28	2912.60	2914.36	5.85
CZS-11	CR	GFLDVIKHVGKAA GKAAALNAV TEMVNAEQ*	- mc, Ed,ms ²	100	104	27	2780.50	2782.26	2.91
CZS-12	CR	GFLDVVKHVGKAV GKAAALNAV NDLVNQA*	- mc, Ed,ms ²	100	81	27	2775.54	2777.22	36.95
CZS-13	CR	GFLDVV - HVVGKAV GKAAALNAV NDLVNa	- mc, Ed,ms ²	100	55	26	2519.39	2519.93	11.76
CZS-14	CR	GFLDVLHVGLAA GKAAALNAV NEAVNQ*	- Ed,ms ²	100	87	27	2703.47	2705.11	1.78
CZS-15	CR	GFLDVIKGVGL VALGAVSK S	- Ed,ms ²	100	40	20	1929.13	1930.32	3.60

a= amidation, mc= molecular cloning, ms2= tandem mass spectrometry, Ed=Edman degradation sequencing, accession numbers: KX065078-KX065088, COHK07-COHK-12.

Table 3. Physico-chemical properties of cruzioseptins 1, 2, 3 from *Cruziophyla calcarifer*.

Peptide	Origin	Sequence/Secondary structure*	Theoretical average mass Da.	Hydrophobicity <H>	Hydrophobic moment <μH>	α-helix (%)	Net charge
CZS-1	ECU	G F L D I V K G V G K V A L G A V S K L F amide c c c e e c c c c c h h h h e e c e e c	2117.60	0.581	0.472	19.05	3.00
CZS-2	ECU	G F L D V I K H V G K A A L G V V T H L I N Q amide c c c c c e e c c c c c c e e e e e e e c c	2428.90	0.563	0.464	0.00	2.00
CZS-3	ECU	G F L D V V K H I G K A A L G A V T H L I N Q amide c c c c c c c c c c c h h h h h h e e e e e c	2400.85	0.523	0.441	30.43	2.00

* secondary prediction based on GOR4: h=alpha helix, c=random coil, e=extended strand, accession numbers KX065078-KX065080.

Table 4. Minimal inhibitory concentrations (MICs) and haemolytic activity of synthetic cruzioseptins from *Cruziophyla calcarifer*.

Synthetic peptide	MIC μM (mg/L)			MBC μM (mg/L)			Ha mM(mg/L)	Species	Ref.
	<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>			
CZS-1	15.11 (32)	3.77 (8)	3.77 (8)	15.11 (32)	7.56 (16)	15.11 (32)	120.87(256)	<i>C. calcarifer</i>	
CZS-2	26.35 (64)	6.59 (16)	13.18 (32)	26.35 (64)	26.35 (64)	52.69 (128)	210.96(512)	<i>C. calcarifer</i>	
CZS-3	13.32 (32)	13.32 (32)	13.32 (32)	26.66 (64)	53.31 (128)	53.31 (128)	213.33(>512)	<i>C. calcarifer</i>	
Dermaseptin-B4	5	3	NA				NA	<i>P. bicolor</i>	32
Dermadistinct-L	2.5	1.3	NA				NA	<i>P. distincta</i>	33

Ha=100% of Haemolytic activity, NA=not available

Figure 1. Nucleotide and translated open-reading frame amino acid sequences of the sense strand of cloned cDNAs encoding cruzioseptins 1 to 9, 11 and 12 from *Cruziophyla calcarifer*. The putative signal peptides are double-underlined, acidic spacers are in italics, the mature peptides are single-underlined and the stop codons are indicated by asterisks. Accession numbers KX065078 and KX065088, respectively.

A) Cruzioseptin-1 (CZS-1). **B)** Cruzioseptin-2 (CZS-2). **C)** Cruzioseptin-3 (CZS-3). **D)** Cruzioseptin-4 (CZS-4). **E)** Cruzioseptin-5 (CZS-5). **F)** Cruzioseptin-6 (CZS-6). **G)** Cruzioseptin-7 (CZS-7). **H)** Cruzioseptin-8 (CZS-8). **I)** Cruzioseptin-9 (CZS-9). **J)** Cruzioseptin-11 (CZS-11). **K)** Cruzioseptin-12 (CZS-12) **L)** Domain structure of the antimicrobial peptide precursors: **1.** putative signal peptide. **2–5** acidic spacer peptides. **3, 5.** propeptide convertase processing sites. **6.** mature antimicrobial peptide. **7.** C-terminal processing site with glycyl G residue amide donor indicated with an asterisk.

Figure 2. Reverse phase HPLC chromatogram of *Cruziophyla calcarifer* skin secretion fractionated over 240 min with dual UV detection at 214 nm (red line) and 280 nm (green line). Arrows denote retention times of fractions with antimicrobial activity. Cruzioseptin-1 was identified in fraction 171 and Cruzioseptin-2 in fraction 162.

Figure 3. Mass analysis of antimicrobial HPLC fraction with retention time 171 min containing Cruzioseptin 1. A) The arrow denotes a singly-charged ion of m/z 2117.54 obtained by MALDI-TOF MS analysis. B) LCQ MS ESI denotes precursor ions of m/z 2+ 1059.75 and 3+ 706.67 corresponding to CZS-1.

Figure 4. Mass analysis of antimicrobial HPLC fraction with retention time 162 min containing Cruzioseptin 2. A) Arrow denotes a singly charged ion of m/z 2427.42 obtained by MALDI-TOF MS analysis B) LCQ MS ESI denotes precursor ions of m/z 2+ 1215.08, 3+ 810.50, and 4+ 316.25 corresponding to CZS-2.

Figure 5. Synthetic cruzioseptins 1, 2, and 3 produced by SPPS and purified by RP-HPLC.

A) Cruzioseptin-1 single charge ion of m/z 2117.63. **B)** Cruzioseptin-2 single charged ion of m/z 2427.38. **C)** Cruzioseptin-3 single charged ion of m/z 2400.61.

Figure 6. LCQ MS/MS Sequencing of Cruzioseptin-1 (A), Cruzioseptin-2 (B), and Cruzioseptin-3 (C). Each table contains the predicted b and y ions from each sequence. Observed ions are underlined in blue and red typefaces.

Figure 7. Predicted alpha helical wheel plots of cruzioseptins 1, 2, and 3. Basic residues are in blue and acid residues are in red. The basic amino acid histidine is in light blue as its charge depends on pH. Non polar residues are in yellow and polar residues are in purple. Uncharged residues of glycine and alanine are in grey and asparagine and glutamine are in pink. The arrow points to the hydrophobic face.

Figure 8. Haemolytic activity of Cruzioseptins 1, 2 and 3.

1 **Figure 1**

A) Cruzioseptin-1

[illegible]

B) Cruzioseptin-2

[illegible]

C) Cruzioseptin-3

[illegible]

D) Cruzioseptin-4

[illegible]

E) Cruioseptin-5

[illegible]

1 . *

2 101 GTTGAGAAAA TGTA AACAG AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA

3 151 AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA

4

F) Cruzioseptin-6

5

6 M A Y L K K S L F L V L F L G L V.

7 1 ATGGCTTACC TGAAGAAATC TCTTTTCCTT GTACTATTCC TTGGATTGGT

8 . S L S I C E E E K R E E E N E E

9 51 CTCTCTTTTCG ATCTGTGAAG AAGAGAAAAG AGAAGAGGAG AATGAGGAGG

10 E· Q E D D D Q S E E K R G F L D V.

11 101 AACAGAAGA TGATGATCAA AGTGAAGAGA AGAGAGGCTT CCTGGATGTA

12 I T H V G K A V G K A A L N A V T .

13 151 ATAACACATG TAGGAAAAGC TGTAGGAAAA GCGGCTTTAA ATGCAGTTAC

14 E M V N Q A E Q *.

15 201 TGAAATGGTA AATCAAGCAG AGCAATAA

16

G) Cruzioseptin-7

17

18 M A K L K K S L F L V L F L G L V.

19 1 ATGGCTAAAT TGAAGAAATC TCTTTTCCTT GTGCTATTCC TTGGATTGGT

20 . S L S I C E E E K R E E E N E E .

21 51 CTCTCTTTTCG ATCTGTGAAG AAGAGAAAAG AGAAGAGGAG AATGAGGAGG

22 V Q E D D D Q S E E K R G F L D V

23 101 TACAAGAAGA TGATGATCAA AGTGAAGAGA AGAGAGGCTT CCTGGATGTA

24 V K H V G K A V G K A A L N A V T .

25 151 GTAAAACATG TAGGAAAAGC TGTAGGAAAA GCGGCTTTAA ATGCAGTTAC

26 . E M V N Q A E Q *

27 201 TGAAATGGTA AATCAAGCAG AGCAATAAAG TTGAGAAAAT GTAAAATCGA

28 251 CAAAAAAAAA AAAAAAAAAA AAAAAAAAAA A

29

H) Cruzioseptin-8

30

31 M A F L K K C L F L V L F L G L V.

32 1 ATGGCTTTCC TGAAGAAATG TCTTTTCCTT GTACTATTCC TTGGATTGGT

33 . S L S I C E E E K R E E E N E E

34 51 CTCTCTTTTCG ATCTGTGAAG AAGAGAAAAG AGAAGAGGAG AATGAGGAGG

35 V· Q E D D D Q S E E K R G F L D V

36 101 TACAAGAAGA TGATGATCAA AGTGAAGAGA AGAGAGGCTT CCTGGATGTA

37 I K H V G K A A G K A A L N A V T .

38 151 ATAAAACATG TAGGAAAAGC TGCAGGAAAA GCGGCTTTAA ATGCAGTTAC

39 E M V N Q G E Q *

40 201 TGAAATGGTA AATCAAGGAG AGCAATAACG TTAAGAAAAT GTAAAATCTA

41 251 ATTACTCTAA GGAGTACAAT TATCAATAAT TGTGCCAAAC CTATATTAAA

42 301 GCATATTGAA CTGATAAAAA AAAAAAAAAA AAAAAAAAAA AAAAA

43

I) Cruzioseptin-9

44

45 K R G F L D V I T H V G K A V G K.

46 1 AAGAGAGGCT TCCTGGATGT AATAACACAT GTAGGAAAAG CTGTAGGAAA

47 . A A L N A V N E M V N Q G E Q *

48 51 AGCGGCTTTA AATGCAGTTA ATGAAATGGT AAATCAAGGA GAGCAATAAC

49 101 GTTGAGAAAA TGTA AATCG AATTGCGCTA AGAAGTAAAA TTATTATTAA

50 151 ACTGAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA

51

J) Cruzioseptin-11

52

53 M V K L K K S L F L V L F L G L V .

54 1 ATGGTTAAAC TGAAGAAATC TCTTTTCCTT GTATTATTCC TTGGATTGGT

55 . S L S I C E E E K R E E E N E E V .

56 51 CTCTCTTTCT ATCTGTGAAG AAGAGAAAAG AGAAGAGGAG AATGAGGAGG

1 · Q E D D D Q S E E **K R G F L D I**
2 101 TACAAGAAGA TGATGATCAA AGTGAAGAGA AGAGAGGCTT CCTGGATATA
3 V K H V G K A A G K A A L N A V T·
4 151 GTAAAACATG TAGGAAAAGC TGCAGGAAAA GCAGCTTTAA ATGCAGTTAC
5 · E M V N Q G E Q *
6 201 TGAAATGGTA AATCAAGGAG AGCAATAAAG TTAAGAAAAT GTAAAATCTA
7 251 ATTACTCTAA GGAGTACAAT TATCAATAAT TGTGCCAAAC CTATATTAAA
8 301 GCATTTTGAA CAAAAAATAA AAAAAAATAA AAAAAA
9

K) Cruzioseptin-12

11 M A F L K K S L F L V L F L G L V·
12 1 ATGGCTTTCC TGAAGAAATC TCTTTTCCTT GTACTATTCC TTGGATTGGT
13 · S L S I C E E E **K R** E E E N E E
14 51 CTCTCTTTTCG ATCTGTGAAG AAGAGAAAAAG AGAAGAGGAG AATGAGGAGG
15 V· Q E D D D Q S E E **K R G F L D V**
16 101 TACAAGAAGA TGATGATCAA AGTGAAGAGA AGAGAGGCTT CCTGGATGTA
17 V K H V G K A V G K A A L N A V N·
18 151 GTAAAACATG TAGGAAAAGC TGTAGGAAAA GCGGCTTTAA ATGCAGTTAA
19 · D L V N Q G E Q *
20 201 TGATTTGGTA AATCAAGGAG AGCAATAAAG TTAAGAAGAT GTAAAATCGA
21 251 ATTGCGCTAA GAAGTAAAAAT TATTATTAAA CTGAGAAAAA AAAAAAAAAA
22 301 AAAAAAAAAA A
23

L)

25 ←-----1-----→ 2 3 ←-----4-----→ 5 ←-----6-----→ 7
26 CZS-1 MAFLKKSFLVLFLGLVLSLSIC *EEE KR EENEEQDDDEQSEE* **KR** GFLDIVKGVGKVALGAVSKLF G* QEER
27 CZS-2 MAFLKKSFLVLFLGLVLSLSIC *EEE KR EEENEVQEDDDQSEE* **KR** GFLDVIKHVGKAALGVVTHLINQ G* EQ
28 CZS-3 MAFLKKSFLVLFLGLVLSLSIC *EEE KR EEENEVQEDDDQSEE* **KR** GFLDVVKHIGKAALGAVTHLINQ G* EQ
29 CZS-4 MAFLKKSFLVLFLGLVLSLSIC *EEE KR EEENEVQEDDDQSEE* **KR** GFLDVIKHVGKAALSVMVSHLINE G* EH
30 CZS-5 MAFLKKSFLVLFLGLVLSLSIC *EEE KR EEENEVQEDDDQSEE* **KR** GFLDVIKHVGKAVGKAALNAVNDMVNKPEQQS
31 CZS-6 MAYLKKSFLVLFLGLVLSLSIC *EEE KR EEENEVQEDDDQSEE* **KR** GFLDVITHVGKAVGKAALNAVTEMVQNQAEQ
32 CZS-7 MAKLKKSFLVLFLGLVLSLSIC *EEE KR EEENEVQEDDDQSEE* **KR** GFLDVVKHVGKAVGKAALNAVTEMVQNQAEQ
33 CZS-8 MAFLKKCLFLVLFLGLVLSLSIC *EEE KR EEENEVQEDDDQSEE* **KR** GFLDVIKHVGKAAGKAALNAVTEMVQNQ G* EQ
34 CZS-9 MAFLKKCLFLVLFLGLVLSLSIC *EEE KR EEENEVQEDDDQSEE* **KR** GFLDVITHVGKAVGKAALNAVTEMVQNQ G* EQ
35 CZS-11 MVKLKKSFLVLFLGLVLSLSIC *EEE KR EEENEVQEDDDQSEE* **KR** GFLDIVKHVGKAAGKAALNAVTEMVQNQ G* EQ
36 CZS-12 MAFLKKSFLVLFLGLVLSLSIC *EEE KR EEENEVQEDDDQSEE* **KR** GFLDVVKHVGKAVGKAALNAVNDLVNQ G* EQ
37

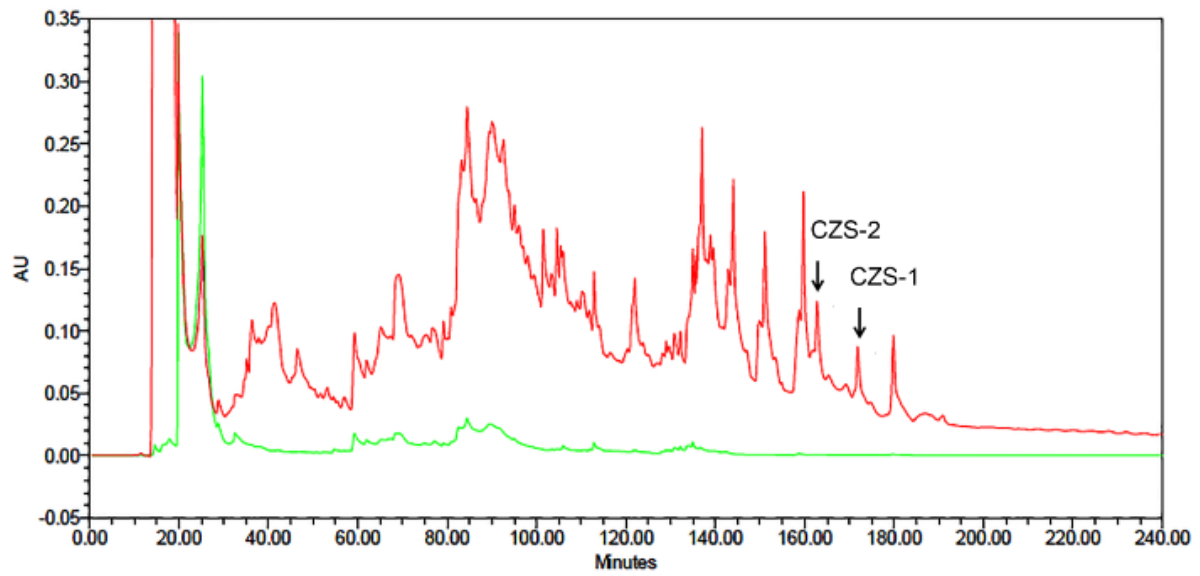
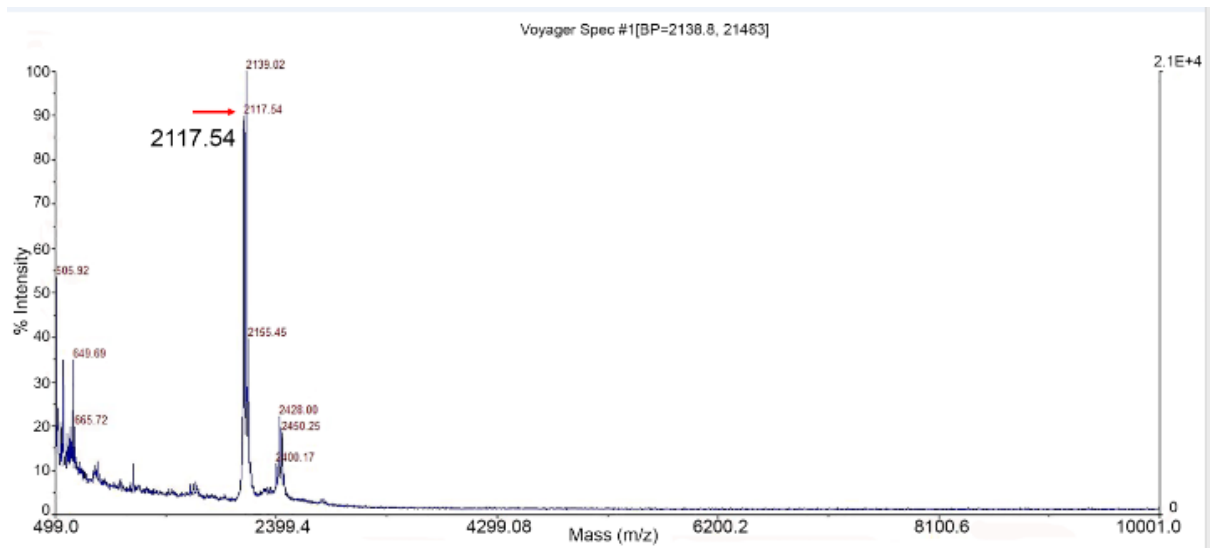
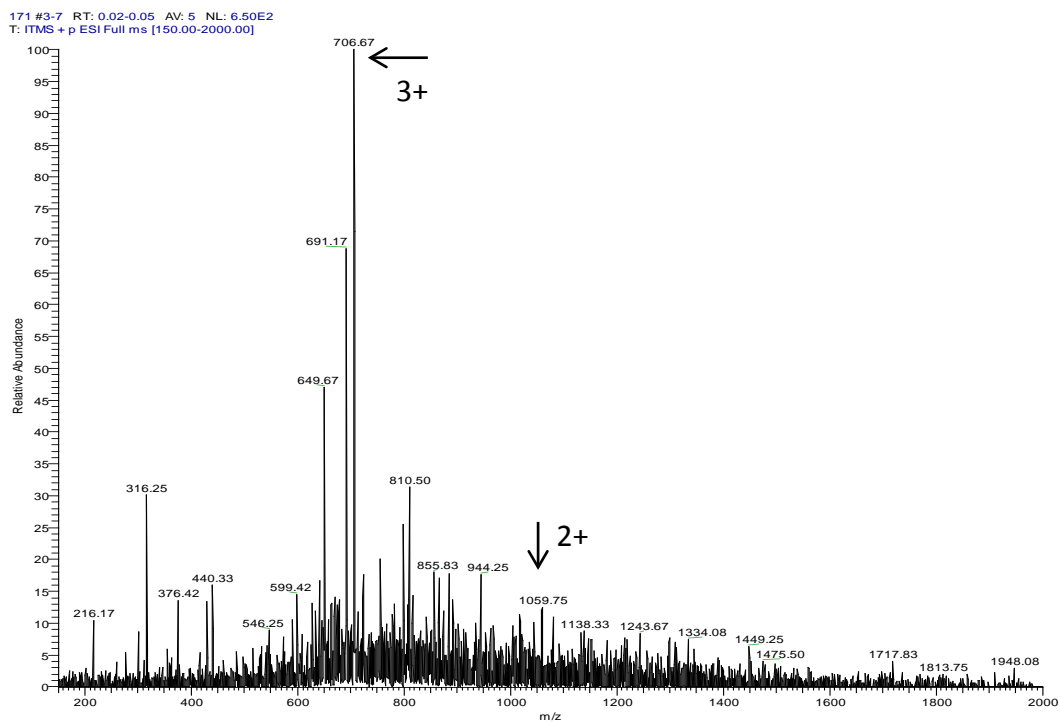
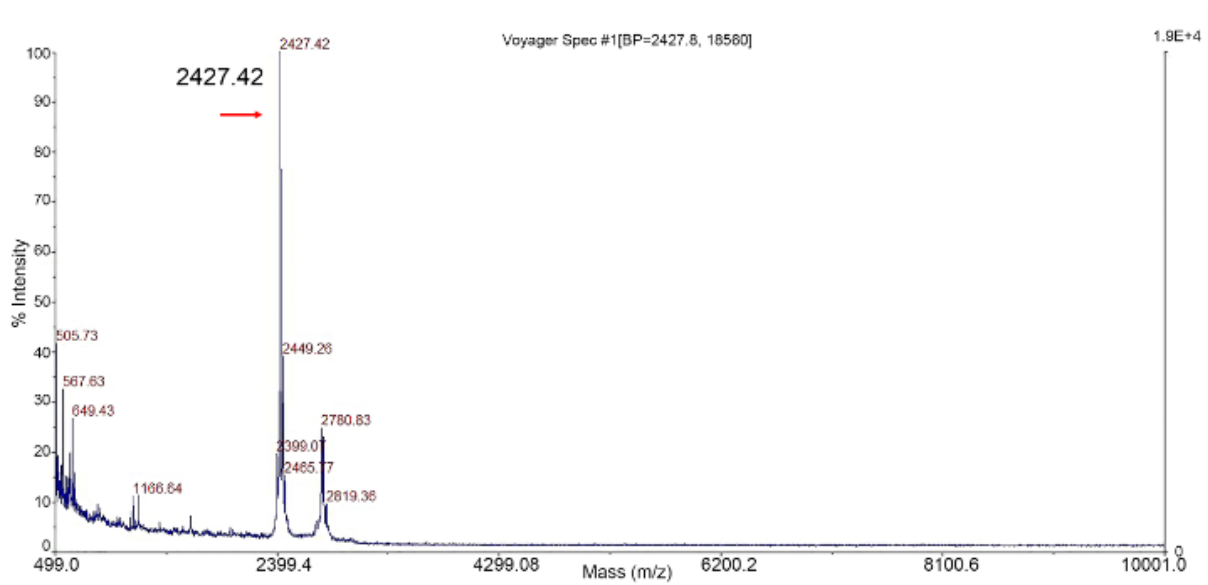
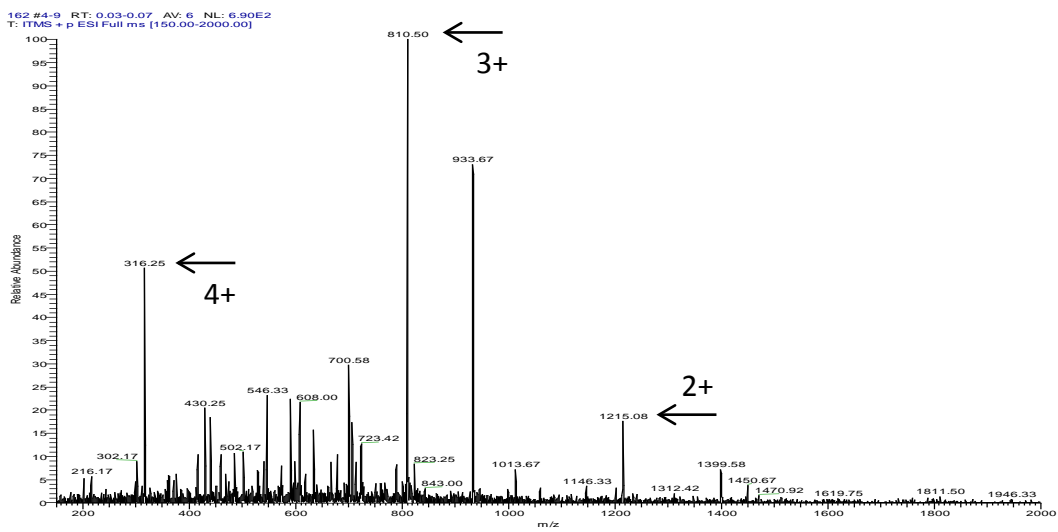
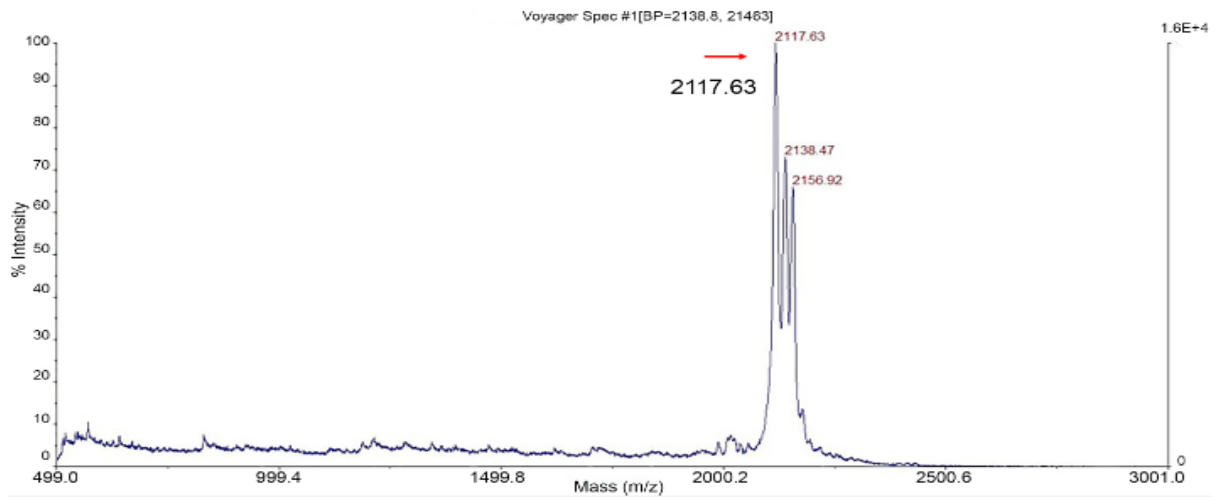
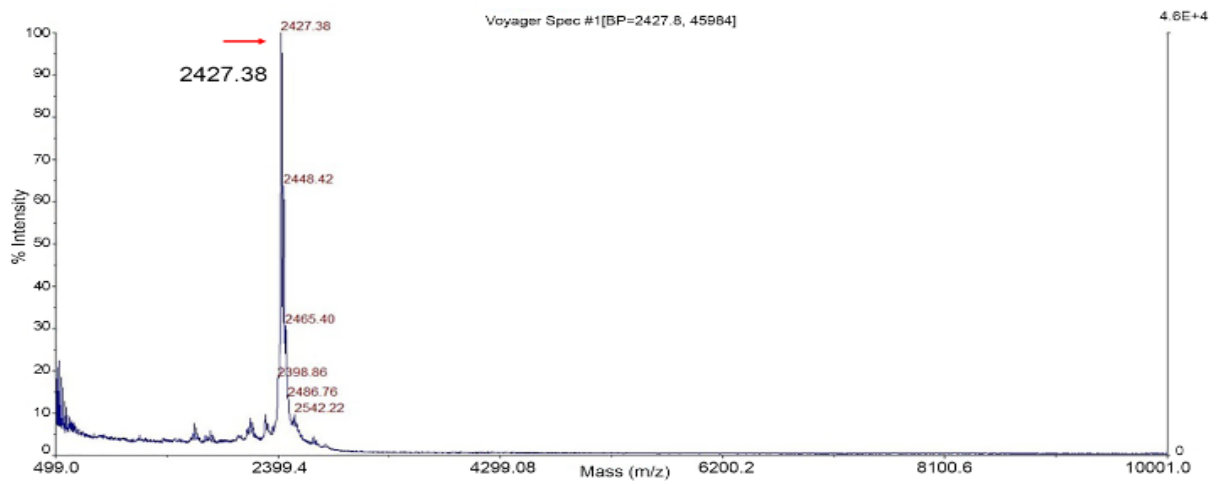
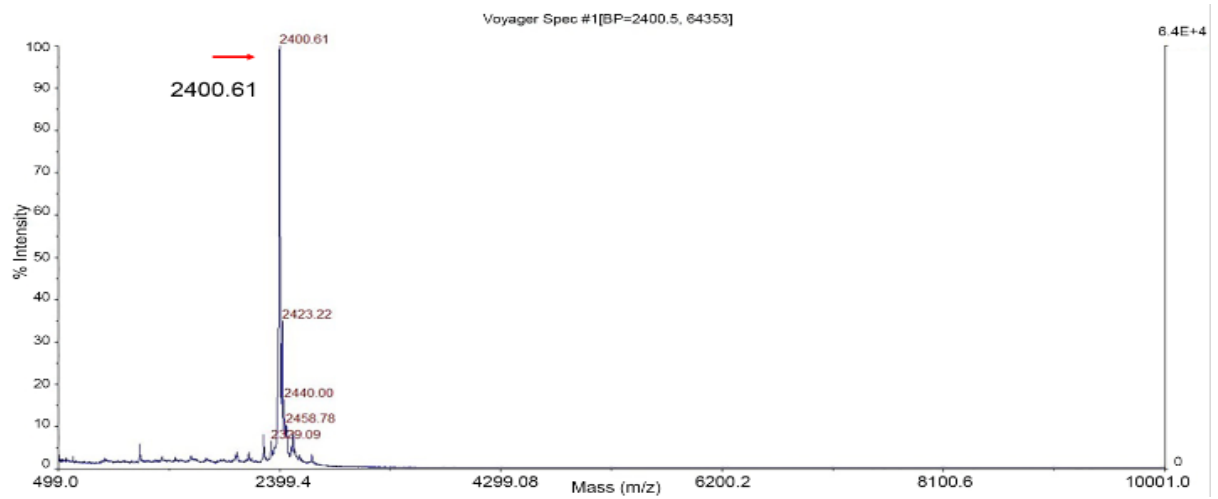
1 **Figure 2**

Figure 3**A)****B)**

1 **Figure 4**2 **A)**4 **B)**

1 **Figure 5**2 **A)**3 **B)**6 **C)**

1 Figure 6

A) Cruzioseptin-1

#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	58.02875	25.51801	G			21
2	205.09717	103.05222	F	2080.26317	1030.63522	20
3	318.18124	159.59426	L	1913.19475	957.10101	19
4	433.20519	217.10773	D	1800.11083	900.55833	18
5	548.28228	273.64977	I	1685.08373	843.04560	17
6	663.36033	332.18393	V	1671.98988	736.50347	16
7	778.43838	389.21148	K	1472.83124	736.98828	15
8	893.51643	415.74220	G	1244.83827	622.92177	14
9	1008.59448	504.29513	V	1237.81490	614.41104	13
10	1123.67253	561.83626	G	1133.74833	594.87833	12
11	1238.75058	587.33463	K	1131.72481	566.36609	11
12	1353.82863	607.36884	V	1093.82894	546.91447	10
13	1468.90668	734.45334	A	804.68162	452.78440	9
14	1583.98473	791.99187	L	833.52440	417.26584	8
15	1699.06278	849.52991	G	720.44033	360.72380	7
16	1814.14083	907.06791	A	663.41886	332.21307	6
17	1929.21888	964.60594	V	592.38174	296.69451	5
18	2044.29693	886.50895	S	493.31332	247.16030	4
19	1840.10580	920.55844	K	408.28128	203.64428	3
20	1953.18967	977.08847	L	278.18632	139.59680	2
21			F- Amidated	165.10225	83.05476	1

B) Cruzioseptin-2

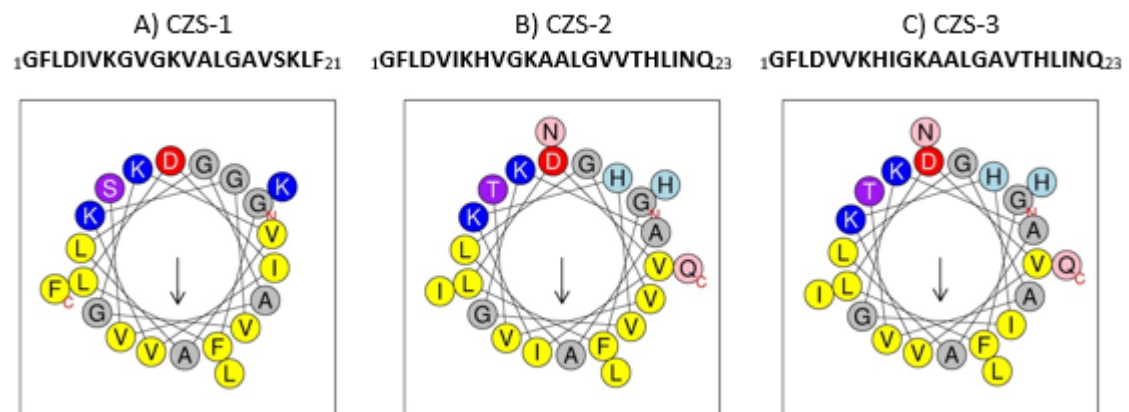
#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	58.02875	25.51801	G			23
2	205.09717	103.05222	F	2371.39736	1186.20232	22
3	318.18124	159.59426	L	2224.32894	1112.88511	21
4	433.20519	217.10773	D	2111.24487	1056.12807	20
5	548.28228	273.64977	V	1996.21792	998.81280	19
6	663.36033	332.18393	I	1897.14950	949.07329	18
7	778.43838	389.21148	K	1784.08543	892.53815	17
8	893.51643	415.74220	H	1655.97046	828.48327	16
9	1008.59448	504.29513	V	1518.91155	759.95841	15
10	1123.67253	533.80586	G	1419.84313	710.42520	14
11	1238.75058	621.85135	K	1362.82166	681.91447	13
12	1265.73654	633.37191	A	1234.72888	617.88893	12
13	1353.82863	681.88047	A	1163.68857	582.34542	11
14	1449.85773	725.43250	L	1082.85245	546.82986	10
15	1506.87920	763.94324	G	979.68833	490.28783	9
16	1621.95725	811.97445	V	822.64881	461.77709	8
17	1737.03530	869.01188	V	724.41007	412.24233	7
18	1852.11335	926.55550	T	624.41007	362.70867	6
19	1943.12263	972.08485	H	523.38238	312.18483	5
20	2056.20670	1029.60888	L	423.30243	243.85533	4
21	2169.29077	1086.14802	I	323.21841	187.11334	3
22	2283.33370	1142.17049	N	280.13534	130.57131	2
23			Q- Amidated	146.09241	73.54884	1

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C) Cruzioseptin-3

#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	58.02875	25.51801	G			23
2	205.09717	103.05222	F	2343.36606	1172.18887	22
3	318.18124	159.59426	L	2196.29764	1098.65246	21
4	433.20519	217.10773	D	2083.21357	1042.11042	20
5	548.28228	273.64977	V	1968.18662	984.68885	19
6	663.36033	332.18393	V	1869.11820	935.08274	18
7	778.43838	380.22364	K	1770.04873	885.62853	17
8	893.51643	448.75309	H	1641.85431	821.48104	16
9	1008.59448	505.29513	I	1504.88580	752.95159	15
10	1123.67253	563.80538	G	1381.81133	696.40955	14
11	1238.75058	621.85135	K	1334.79036	667.88832	13
12	1265.73654	633.37191	A	1208.88538	603.85133	12
13	1353.82863	681.88047	A	1135.85827	568.33277	11
14	1449.85773	725.43250	L	1084.82115	542.81421	10
15	1506.87920	763.94324	G	961.63708	476.27218	9
16	1577.81832	789.48130	A	834.61581	447.76144	8
17	1678.88474	838.98801	V	724.41007	412.24233	7
18	1779.95242	889.51935	T	624.41007	362.70867	6
19	1880.02033	938.04830	H	523.38238	312.18483	5
20	2028.17540	1014.68134	L	423.30243	243.85538	4
21	2141.25947	1071.13337	I	323.21841	187.11334	3
22	2255.30240	1123.15434	N	280.13534	130.57131	2
23			Q- Amidated	146.09241	73.54884	1

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1 **Figure 7**

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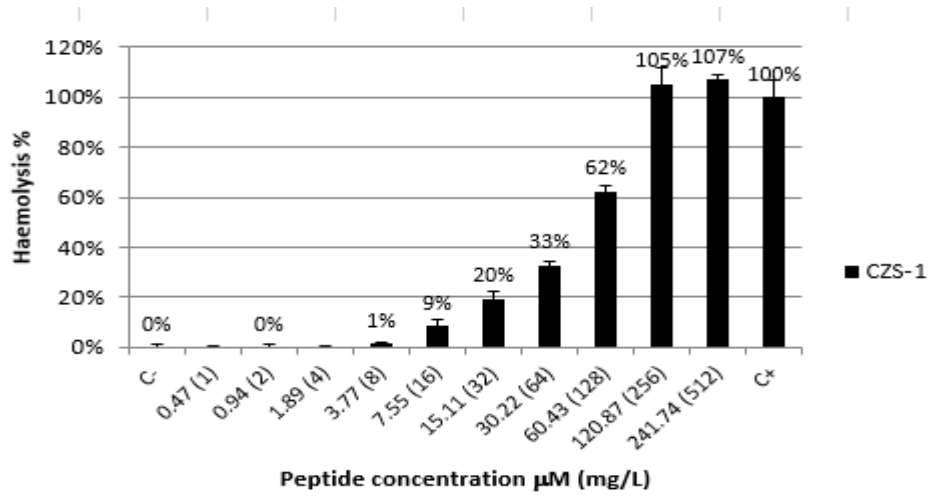
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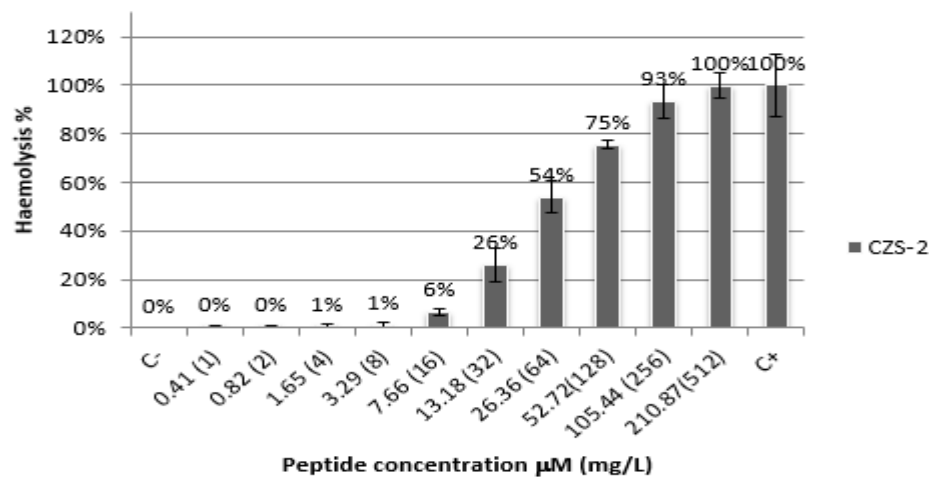
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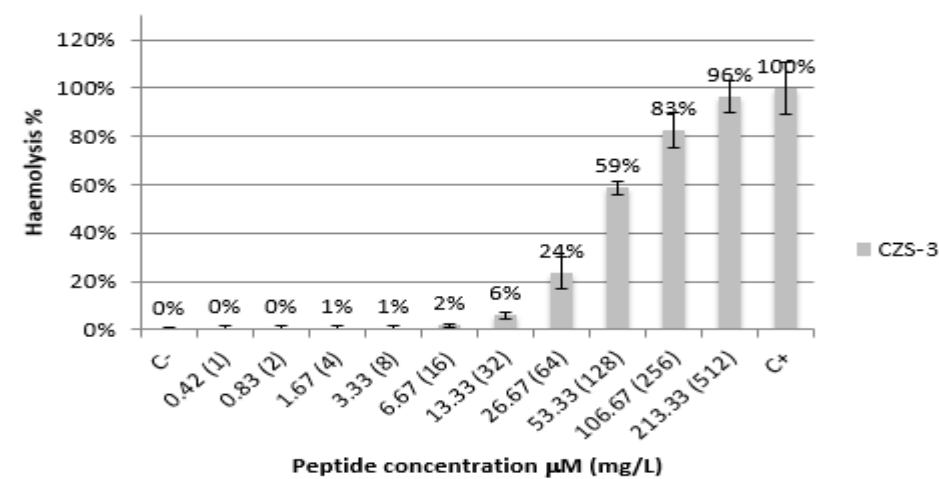
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1 **Figure 8**2 **A)**

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4 **B)**

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6 **C)**

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